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IN MALE HOLTZMAN RATS

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BEHAVIORAL AND PHYSIOLOGICAL EFFECTS OF LATE-ONSET PROTEIN RESTRICTION IN MALE HOLTZMAN RATS

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ABSTRACT

BEHAVIORAL AND PHYSIOLOGICAL EFFECTS
OF LATE-ONSET PROTEIN RESTRICTION
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Certain populations of older people are affected by late-onset dietary stress coupled with behavioral changes. Using an animal model twenty, 200 day-old rats were placed in pairs by similarity of body weight with one pair-member receiving a low-protein (3 percent) diet and the other an adequate-protein (24 percent) diet. Weights were monitored, plasma protein levels determined and tests carried out for: 1) activity level, 2) exploratory behavior, 3) food preference, 4) motor-coordination and 5) learning.

Protein levels and weights were significantly greater for controls as were results of motor-coordination testing. Fifty percent of the low-protein rats became emaciated. Exploratory behavior was significantly greater for the adequately nourished animals. Low-protein rats made more errors on oddity discrimination learning but differences were not significant. Both groups significantly preferred the adequate-protein diet when both were offered simultaneously. Results suggest that behavioral and physiological changes in rats can result from protein-deficient diets.

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INTRODUCTION

Numerous studies have addressed the problem of the detrimental effects of dietary deficiency on various parameters. Certain investigators using animal models have been particularly interested in behavior and learning manifestations resulting from protein-calorie deficient diets. The impetus for much of the early research on the effect of protein-calorie malnourishment on physical growth, learning and behavior was the identification of the health problem, kwashiorkor, prevalent in Africa and other underdeveloped areas. Signs and symptoms of kwashiorkor include irreversible stunting of physical growth, irritability and retarded mental development (Levitsky and Barnes, 1973). Factors influencing the early "critical period" of brain development are believed to be the primary cause of this latter problem and therefore studies have involved dietary manipulation during the perinatal period.

Physiological Findings

Studies on the effects of protein deprivation on immature laboratory animals have been carried out by many investigators using a variety of techniques to effect malnutrition and investigate its consequences. Richter (1959) described a higher rate of amino acid incorporation into the proteins of the developing newborn brain of the rat. Laboratory investigations by Chow (1964) revealed that dietary restriction of rat dams during pregnancy and lactation or during lactation alone resulted in significant reduction in body growth in pups. This was permanent for rats malnourished during the combined *in utero* and suckling period and was the same for male and female pups. This study was a relatively early one in this area of research and approached the methods of feeding not from administration of carefully prepared laboratory diets of differing, but well characterized, protein-calorie concentrations but instead by use of a commercial laboratory feed which was restricted in the amount given to some animals. This is a major criticism when studies require diet manipulation. Ingredients of commercially prepared diets, when analyzed, have been found to be inconsistent with reported content information (Greenfield and Briggs, 1971).

Mouse models were used by Randt and Derby (1973) who found that severe nutritional restriction of the dams during gestation and suckling resulted in mice with mean brain weights that were 20 percent lower at birth than for pups of dams fed normal diets. This developmental defect was noted to be persistent when it was found that the weights of the cerebellum of malnourished rats had a mean of only 86 percent of those of the control when studied at 600 days of age.

Goodrick (1974) fed male mice diets containing 4 percent protein (casein) or 24 percent protein (casein) at five weeks of age and varied the experimental conditions by housing one, two, or five mice per cage. For all groups, the body weights of mice fed normal-protein diets were greater for group-caged than for isolated mice and body weights of mice fed low-protein diets were less for group-caged than for isolated mice. At 15 and 16 weeks of age those mice fed low-protein diets engaged in more exploratory behavior and were less emotional than mice fed normal-protein diets. These results point out the necessity of keeping the possibility of social-behavioral factors in mind when interpreting data from this type of study.

Sobotka et al. (1974) fed lactating dams a 12 percent casein diet while controls received 24 percent casein. The malnourished pups born to the 12 percent dams demonstrated signs of retarded physical development including reduced body growth and delayed eye opening. Rat pups had reached only 44 percent of the body weight of the pups from control litters at the time of weaning. Detailed biochemical analyses of brains from these animals showed a marked reduction in cholesterol, DNA and acetylcholinesterase.

Behavioral Studies

Behavioral changes described in humans suffering from kwashiorkor include apathy and irritability (Cowley and

Griesel, 1964). Several workers have also looked for behavioral effects of protein deficiency in laboratory animals. Cowley and Griesel (1964) studied the maternal behavior of the protein deficient parent generation of rats. No significant behavioral changes in the protein deficient group were observed except in nest building which was more active than with control rats and in time for returning pups to the cage when placed on a retrieval board where the mean time was longer for the low-protein dams. Studies of the first filial generation (male animals) revealed that the protein deficient rats hoarded fewer pellets than the control group and were slower in emerging from their home cages. Differences between protein deficient and control rats were marked on Hall's open field test in that the mean ambulatory score for the low-protein rats was lower and the mean number of fecal boluses was greater when compared to controls.

Frankova and Barnes (1968) continued the same line of investigation with emphasis on behavior. They reported that striking behavioral differences developed in groups of rats which had been restricted in protein both before and after weaning. During the 12 days of the experiment, increased excitability was noted in these animals. Behavioral abnormalities were manifested as inadequate, stereotyped movements and an inability to delay or extinguish fixed conditioned reactions. Those animals with deficient protein intake in the pre-weaning period did not show similar behavior patterns seen in the rats restricted both before and after weaning.

Frankova (1973) studied the various social interactions of malnourished young male rats in an observation cage. The malnourished group had a delayed development of social behavior. On the 49th day, control rats showed increased exploration of the social stimulus, e.g., a second rat was placed in the cage, whereas the malnourished rats were markedly inhibited. It was suggested that the disturbances seen in early social activities were caused by the impaired development of neurosensory structures secondary to the malnutrition and that, not only is there a delay in behavioral development, there is a massive disturbance of the social characteristics of the individual. The protein-calorie deficient rat tries to avoid the new social exposures that the normal animal seeks. Decreased investigative behavior has also been noted in monkeys (Strobel and Zimmerman, 1971). In a free operant chain manipulation situation, protein deficient monkeys showed a marked decrease in responding when novel objects were attached to the chains, whereas performance increased in the adequately nourished control group.

Pettus et al. (1974) as part of the extensive primate research program at the University of Montana, investigated the behavior of protein-malnourished monkeys and concluded that the behavioral syndrome associated with protein-calorie malnutrition may be improved by six months of nutritional rehabilitation. Peregoy et al. (1972) found that previously protein-malnourished rhesus monkeys showed a marked preference for high protein food when compared to adequately nourished controls.

Barnes et al. (1968) studied postnatal nutritional deprivations in rats to determine the effects on adult behavior toward food and found total food intake was significantly greater in the malnourished group compared to the controls. When food was available for only one hour each day, male rats fed the low protein diet had a three fold increase in food spillage. There were no significant differences in either food consumption or food spillage in female rats when compared to controls.

Learning Studies

Barnes et al. (1966) deprived rats of food during the first three weeks of life by increasing the number of rat pups nursing one dam. Control litters contained eight pups each. These animals were weaned at three weeks of age and fed a low-protein diet (4 percent casein in one group and 3 percent in the other) for eight weeks. The four groups used were: 1) control, 2) nutritional deprivation by increasing the number of pups nursing, 3) nutritional deprivation by feeding a low protein diet after weaning, and 4) a combination group in which both procedures were used to produce malnutrition. Males deprived prior to and after weaning made significantly more errors in a Y-water maze test of visual discrimination than did females. This is the only study of which we are aware reporting a sex difference on a learning measure. These authors found that nutritional deprivation in early life can cause a long lasting, possibly permanent retardation in the development of learning behavior. They concluded that motivational or emotional behavioral differences noted among the treatment groups made it difficult to know the relative contribution of drive versus capacity in the later learning behavior.

Rats maintained on a low-protein diet from the time of weaning made fewer errors than controls in a multiple-Y maze when the reinforcement was protein-adequate food (Griffiths and Senter, 1954). This study is of special importance since it demonstrated that when food is used as an incentive for learning in nutritional deprivation studies, the malnourished animals may be more motivated to perform. This consideration has been overlooked in previously reported studies where food was used as the reinforcement (Cowley and Griesel, 1959 and 1963).

Several studies using the protein-deficiency model have resulted in different findings concerning the time of greatest vulnerability to dietary stress in later adult performance on learning tasks. Giurintano (1974), using Wistar rats, found that postnatal protein deprivation resulted in deficiencies in learning a difficult water maze. No such deficits were found when dams of another group of subjects were protein-deprived during gestation. No learning deficiencies were found in a study by Frankova and Barnes (1968) in avoidance conditioning learning, when subjects were both pre- and postnatally malnourished. Bush and Leathwood (1975) using Swiss white mice found poorer performance in avoidance learning tasks for both the pre- and postnatally malnourished groups, when compared to controls. The species used, and the type and complexity of the learning task may be important considerations when findings in the literature fail to agree.

Levitsky and Barnes (1973) have delineated the striking similarities between the effects of early malnourishment and environmental stimulus deprivation and have suggested that the underlying mechanisms causing these physiological and behavioral changes may be similar. These investigators found that when both diet and environment were manipulated, negative behavioral effects increased, suggesting an interaction between these two variables (Levitsky and Barnes, 1972).

Review of the above literature indicates that pre- and postnatal dietary stress has detrimental physiological and behavioral consequences in laboratory animals and these effects appear to be analagous to those present in children afflicted with kwashiorkor. Conclusions drawn from many of the early studies are questionable since serious methodological problems, as mentioned above, make the findings difficult to interpret. Consideration of the overall body of literature in this area, however, indicates that perinatal malnutrition does adversely affect the organism, even though these effects are inconsistent among studies and are often poorly defined.

Nutrition and Longevity

The effects of diet on longevity have been studied in rats by several investigators using protein and/or calorie restriction models. McCay (1939) found that young rats fed diets, restricted in calories only, experienced retarded growth and increased life span. The rats were restricted for 300, 500, 700 or 1,000 days before growth to maturity was allowed. Rats from each of the four groups were living when the last of the controls had died. McCay suggested that this method could be used to produce very old animlas for aging studies.

A study was designed to determine if intermittent fasting in rats would cause longer life when compared to controls fed *ad libitum* (Carlson and Hoelzel, 1946). The three experimental groups were fasted as follows: one day in four, one day in three or one day in two. The mean life spans of all three groups exceeded that of the control group and fasting one day in three was optimum. In contrast to McCay's study little or no physical stunting occurred using this intermittent fasting model. Carlson and Hoelzel (1948) used an intermittent fasting design combined with low residue or bulky diets and found that bulk-formers added to diets fed *ad libitum* resulted in increased life spans similar to those found when rats were fasted one day in three. High fat diets of 22.7 percent fat content caused a significant decrease in life span in both male and female rats when compared to a group fed a carbohydrate diet containing 3.4 percent fat (French et al. 1953). The same rate of growth occurred in both experimental groups even though fewer calories were consumed in the high fat condition.

The effects of nutrition on longevity and onset of disease were studied by Berg and Simms (1960). There were three levels of food intake: 1) unrestricted, *ad libitum*, 2) 33 percent less than *ad libitum*, and 3) 46 percent less than *ad libitum*. Retarded growth was avoided by these less severely restricted diets and only rats free from infection were used so that the possibility of confounding nutrition and infection variables would be minimal. Condition one (*ad libitum*) produced rats with large skeletal size and obesity. No obesity occurred in the other two conditions (33 and 46 percent), little skeletal retardation was noted, and onset of disease was delayed as evidenced by postmortem autopsies and microscopic tissue examination. The life span was extended (Berg and Simms, 1961) and the rats living beyond 800 days were studied for the effects of restricted diet on mortality rate, onset of disease, appearance and behavior. The restricted group, when compared to the unrestricted controls, weighed 40 percent less.

They were not, however, malnourished and when over 1,000 days old had the appearance of young, healthy rats. They were more energetic than the obese *ad libitum* animals who developed weakness or paralysis of the hind legs and loss of tail movement. The restricted condition continued the lower mortality trend found in the previous study, and this was attributed to the later onset of organ lesions.

Most research on the relationship between diet and longevity has used lifelong dietary regimens. The underlying assumption was that if the nutritional needs were not met during the growing period, late-onset diet changes would not influence life span. Ross (1972) studied the effects of changing the types and amounts of food intake in older male rats and found that the level of caloric restriction and the protein-calorie ratio of various diets either lengthened or shortened life expectancy. Longevity increased in rats fed a limited diet with a protein-calorie ratio of 1:5. This regimen of food intake caused a reduction in body weight. When other groups of animals were fed diets that increased body weight, the length of life was found to decrease. A program of life-long restricted food intake had a greater effect on longevity, however, even in the adult life of the rat the length of life was significantly increased through dietary manipulation.

Nutrition of the Elderly

Many complex factors are involved in the nutrition of the elderly human and in the relationship of diet to the aging processes. Nutritional status of the elderly may be influenced by prior cultural sets and decreased physical mobility and vigor so that food behavior is adversely affected. Malabsorption can be related to dental problems, loss of gastric and enteric function, and diminished income for food (Mann, 1973, Loeb and Howell, 1970a). Some aged persons suffer from senile anorexia with complaints including burning of the tongue, dry mouth, loss of taste and difficulty in swallowing. When frequently associated diseases presenting with these symptoms, such as carcinoma, uremia and chronic infections, are ruled out the remaining majority of cases are left undiagnosed as to cause. Vinther-Paulsen (1952) evaluated the food intake of elderly patients (67 to 96 years) and concluded that senile anorexia may be due in some cases to malnourishment. Many patients had a history of insufficient nutrition prior to the onset of symptoms.

Loeb and Howell (1970b) summarized the relationship between deficient diet and psychological performance. The symptoms of subclinical forms of specific and general dietary deficiency are: 1) loss of appetite, 2) fatigue, 3) irritability, 4) anxiety, 5) recent memory loss, 6) insomnia, 7) distractability, and 8) mild delusional states. They pointed out that many of the symptoms have been reproduced in human experimental research where adult men were deprived of B-complex vitamins for 60-90 days. This was found to result in confusion and lowered judgment. The same picture can be seen with older people on poor diets or with psychiatric syndromes such as in schizophrenics who eat poorly. Furthermore, these researchers (Loeb and Howell, 1970c) pointed out that, in addition to disease, the ingestion of drugs (prescription and non-prescription), alcoholism and depression may influence nutrition in the elderly.

Clinical observations of nutritional deficiency in the aged have rarely been supported with research data. Corless (1973) reviewed the hospital admissions of elderly persons in England. He stated that the precise nutritional needs of old people are unknown but did report that malnutrition in one form or another is very common in hospitalized individuals. An editorial in the British Medical Journal (1974) stated that although severe malnutrition was uncommon in the elderly, certain individuals were clearly at risk. These included people with arthritis who are unable to shop or cook or confused females who may be able to cook for their husbands and yet will not eat themselves.

One of the largest nutritional studies in the elderly was carried out in Russia with 27,181 individuals, 80 years and older (Chebotaryov and Sachuk, 1964). The majority of these individuals ate moderately and were found to be lean on physical examination. Few other details are given except that few vegetarians or consumers of large amounts of meat were found in the group. Schlenker et al. (1973) stated that no good longitudinal study has been carried out, and further noted that completely satisfactory criteria for determining optimum nutritional status are not available.

Kelly et al. (1957) studied females 40-80 years of age, and upon reevaluation seven years later found mortality to be greater when dietary intake contained 40 percent or less than the recommended daily allowance (RDA) for one or more nutrients.

Whanger and Busse (1975) working with psychiatric disorders in the aged, reported that over 70 percent of hospital admissions were eating doubtfully or grossly inadequate diets. Fifty percent of these patients had borderline to low plasma levels of folic acid, and 12 percent had borderline to low levels of vitamin B_{12}. They noted that these two dietary deficiencies have been associated with certain psychiatric disorders, and emphasized the problem of determining in these psycho-geriatric patients, whether the deficiencies cause the illness or result from it.

Previous studies on behavioral effects of malnourishment have been primarily concerned with perinatal nutritional dietary stress, following the discovery of the relationships of diet to kwashiorkor. Studies on diet and longevity have mostly involved life-long dietary programs and have not been specifically concerned with behavior.

The available information on human nutrition among the elderly suggests the need for well controlled, systematic studies to determine the relationship between diet and behavior when the nutritional deprivation occurs in later life.

Use of animal models allows for more careful control of the relevant variables and is of particular value where severely deficient diets are deliberately imposed upon an organism so that the effects can be evaluated. The Holtzman rat strain was selected for this study since its life expectancy is less than other strains. Semi-purified diets were used so that diet composition could be monitored carefully throughout the study. The selection of tests was made on the basis of anticipated changes in the animals, resulting from the experimental condition. Each can be related to the expected differences between the protein-restricted group and the controls as follows: 1) activity wheels to assess general activity, 2) the open field was used to determine if exploratory behavior would be affected by low-protein intake, 3) the rotarod was used to determine if motor-coordination differed between the groups since it was believed that the low protein group would experience loss of muscle mass, 4) the food-preference test was used to demonstrate the presence of sensory capacity in older animals to distinguish between diets of different compositions and to test for possible "specific hungers" in animals on restricted diets, 5) the oddity discrimination task was selected as the test used to find the presence of learning and/or performance differences between the groups. This is a complex task and would thus remove the possibility present when using simpler learning tasks where all animals may perform well, and possibly differences would not be detected (Bachelder and Denny, 1976).

Initially, a protein model of dietary restriction was used since protein foods are the most expensive in the diet and older people may have more difficulty with consumption of protein foods such as meats (e.g., presence of dental problems) and milk products due to lactose intolerance.

The specific aims of this study included the following:

1. To establish an animal model that could be used to evaluate behavioral effects of late-onset dietary stress.
2. To evaluate behavior in aging rats using tests for exploratory behavior (open-field), motor-coordination (rotarod) and learning (oddity discrimination).
3. To evaluate age-appropriateness of selected tests.
4. To define problems associated with using older animals in nutrition-behavior studies.

The general hypothesis tested was that late-onset dietary stress in rats can result in physiological, behavioral and learning differences when compared to adequately nourished controls. Past workers in the area of the relationship between nutrition and behavior, have generally stated that it is unknown in nutrition-behavioral situations whether malnutrition is the cause of psycho-social problems or results from them.

The results of testing the above hypothesis would demonstrate that changes observed in older living organisms can be attributed primarily to experimentally manipulated diets.

The specific hypotheses tested were as follows:

1. Plasma protein levels could be lowered in older rats fed a low protein diet.
2. Weights of low-protein fed animals would be lower than pair-members fed an adequate protein diet, even though the diets were made isocaloric and caloric intake for pair-members was approximately equal.
3. Performance on a motor-coordination task would be poorer in protein restricted animals compared to controls fed adequate amounts of protein.
4. Activity levels would be greater for the well-nourished controls than the experimental group.
5. Exploratory behavior would be greater in the controls compared to the experimental group.
6. Rats fed a low-protein diet would show a preference for an adequate diet when given both to choose between.

7. Adequately nourished rats would do better on a complex learning task, when compared to rats fed protein-restricted diets.

METHOD

Subjects and Diets

Twenty, 200 day-old male Holtzman rats were placed in pairs by similarity of body weight. The weight range for all *Ss* was between 495 and 568 grams, and between zero and four grams for matched pairs. One of each pair was randomly selected to serve as the experimental 3 percent, low-protein *S* and the other pair-member was the 24 percent adequately nourished control (see Table 1 for composition of diets). The *Ss* were pair fed as described by Yang and Mickelsen (1972), where the experimental rats were fed *ad libitum* and the controls fed the amount eaten by their pair-member the previous day. Tap water was available *ad libitum* for all *Ss* and the laboratory was on a 12:12, light:dark cycle.

Apparatus

Testing equipment included: 1) standard activity wheels, adapted for larger rats (Figure 1), 2) a square open-field with 64, six inch squares, enclosed by a 12" high plywood wall, 3) a rotarod motor-coordination apparatus, consisting of a sandpapered drum (6" in diameter) mounted on a rod attached to a motor that could be run at three speeds, 12, 20 or 30 rpm (Figure 2), and 4) an oddity-discrimination apparatus with a start box, choice area and

Table 1. Composition of Diets

3% Protein (Grams)		24% Protein (Grams)
3.35	Casein (90% Protein)	26.70
0.05	Methionine (1.5% of Casein Level)	0.40
4.00	Minerals (Bernhart-Tomarelli Salt Mixture)	4.00
0.40	Vitamins	0.40
0.20	Choline Chloride	0.20
5.00	Fat (Mazola Corn Oil)	5.00
5.00	Cellulose	5.00
82.00	Glucose	58.30
100.00 (Total)		100.00 (Total)

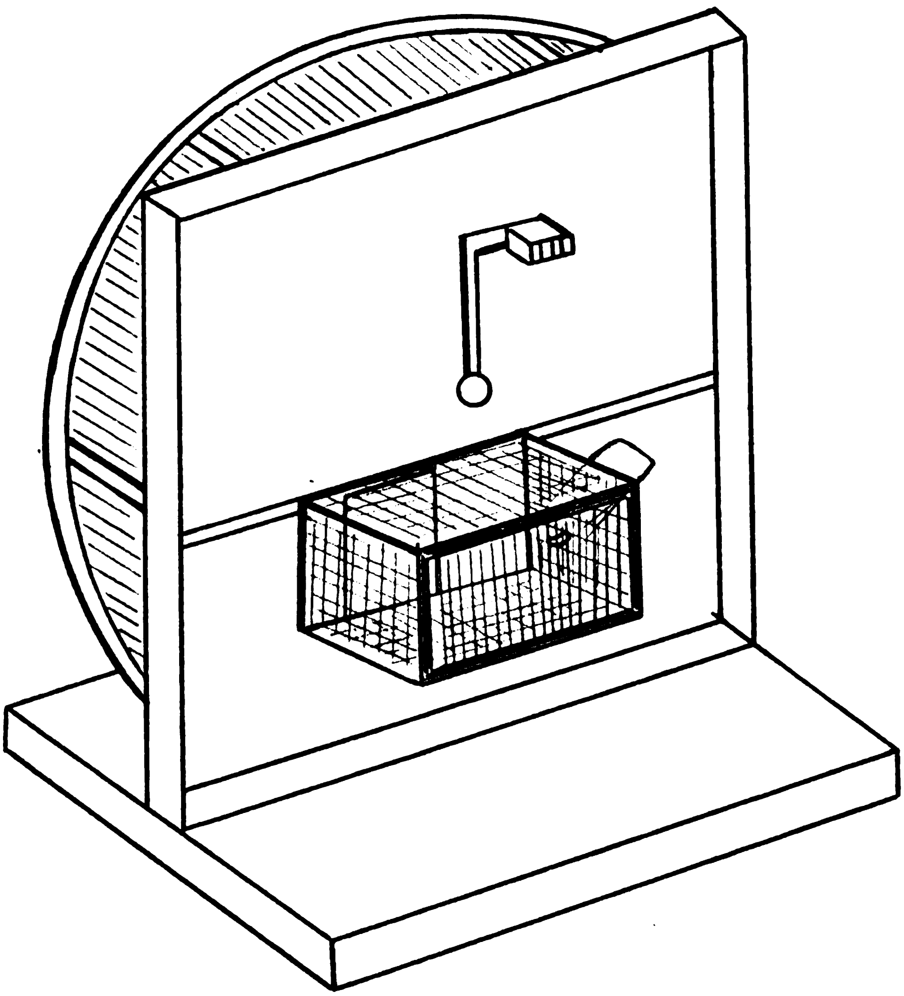

Figure 1. Activity wheel with digital counter. The attached cage can be closed off from wheel by sliding to the left.

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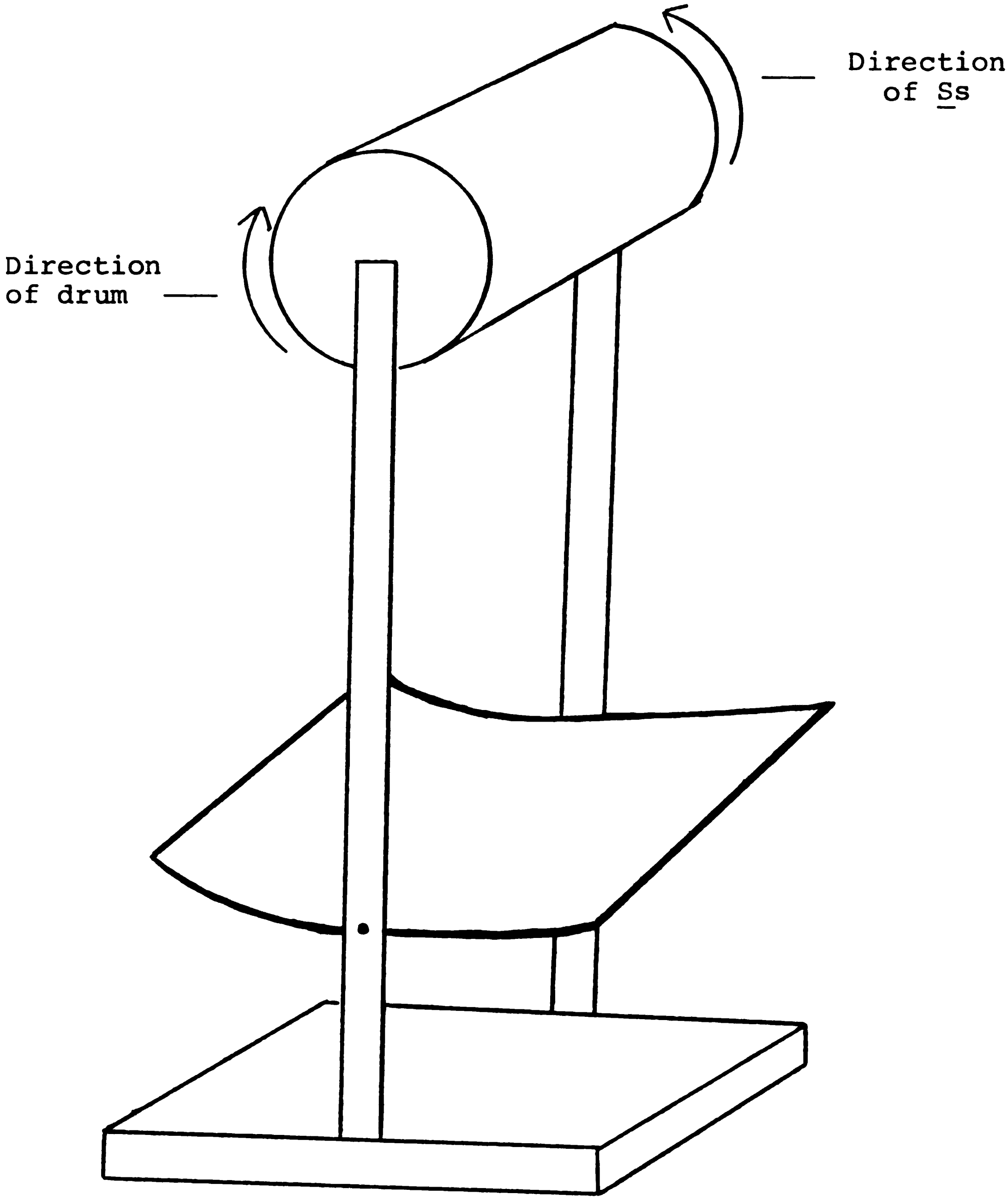

Figure 2. Motor-powered rotarod with sandpaper-covered drum (6" in diameter) and a catch net located below. The drum can be rotated at 12, 20 or 30 rpm.

end chamber. Four removable boxes, two black and two white, were inserted three at a time in various combinations, e.g., WBB, BWB, WBW (Figure 3). All Ss were housed in individual, wire-bottomed cages (8x8x10).

Procedure

All Ss were pair-fed and weighed weekly for 10 weeks. Each S was then etherized and a tail blood specimen taken. Plasma protein levels were determined using the procedure described by Lowry et al. (1951). Following the blood analysis, testing was begun.

Test 1, General Activity: The Ss were approximately 300 days old at the beginning of this test and had been under the pair-feeding condition for 100 days. Ten activity wheels were available and, therefore, the 10 experimental and nine control Ss were randomly assigned to one of two groups. The first group of Ss was housed and fed in activity wheels for eight days. Food and water were available during the 12 hour light cycle and access to the wheel only during a 12 hour dark cycle. The number of revolutions for each S was recorded at the end of each 12 hour period. This procedure was followed for the second group immediately after testing group one.

Test 2, Exploratory Behavior: The Ss were approximately 320 days old at the time of testing, had been under the pair-feeding condition for 120 days and were all tested

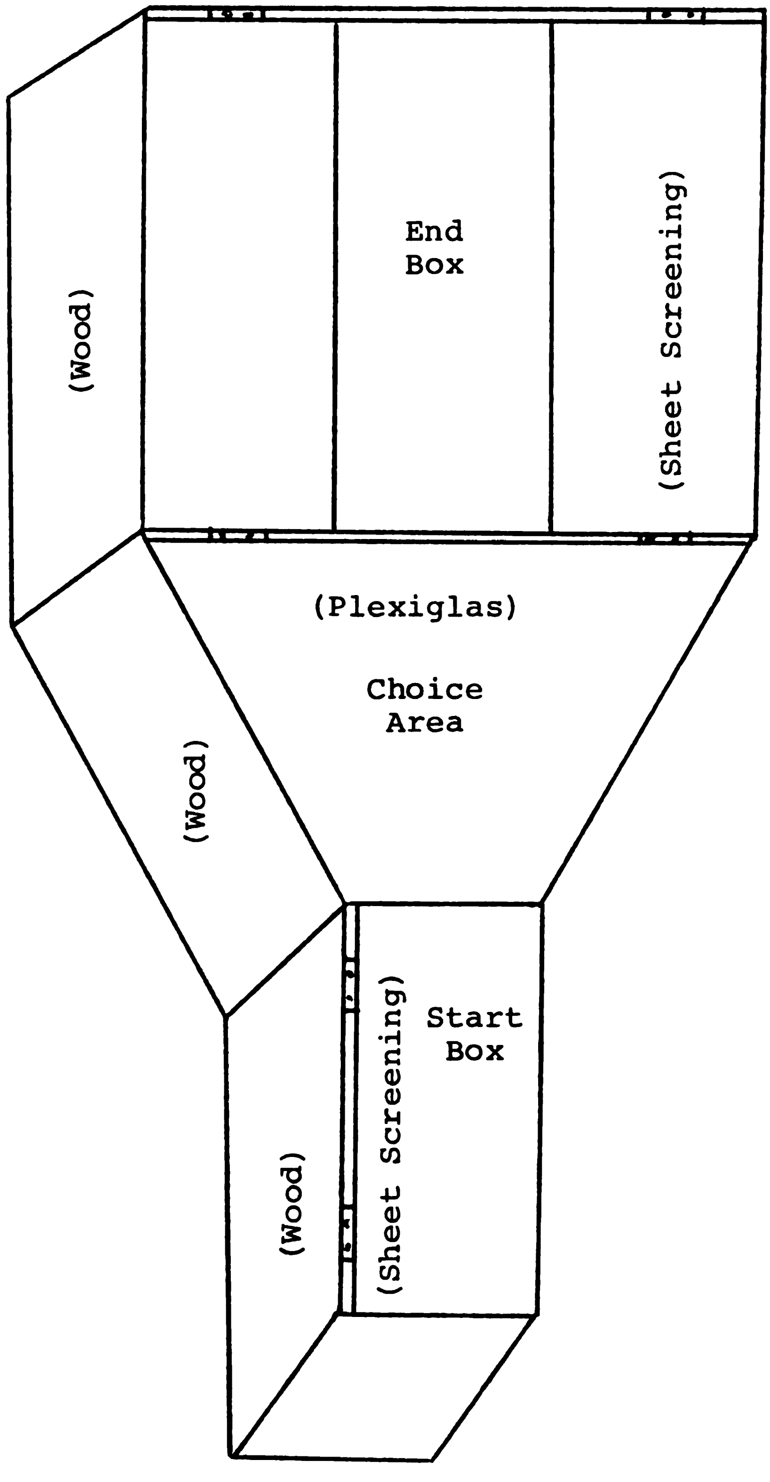

Figure 3. Oddity discrimination apparatus. Four removable boxes, 2 white and 2 black, can be inserted 3 at a time in various combinations. Each box has a door of the same color that can be inserted (not shown).

on the same day. There were nine experimental and seven control _S_s tested. Each _S_s was placed in the corner of the opern-field, facing the vertical line and observed for three minutes. The total number of squares and the number of internal squares entered were recorded.

Test 3, Food Preference: The _S_s were approximately 327 days old and had been pair-fed for 127 days. Eight experimental and seven control _S_s were tested. Two food cups, one containing the 3 percent protein diet and the other the 24 percent protein diet, were placed in each cage and fastened to prevent movement. The cups were rotated from side to side (to control for position bias) for four successive days. The amount eaten from each cup was recorded at the end of each 24 hour period.

Test 4, Motor-Coordination: The _S_s were approximately 350 days old and had been pair-fed for 150 days at the time of testing. Eight experimental and seven controls were tested and the procedure was completed in one day. Each _S_ was tested on the rotarod drum as described by Overmann (1976). There were three practice trials on the drum rotating at 12 rpm, followed on the same day by three test trials, one each at 12, 20 and 30 rpm. The number of seconds that the _S_ remained on the drum was recorded.

Test 5, Oddity-Discrimination Learning: The habituation to the apparatus was begun when the _S_s were 450 days old and had been under the pair-feeding condition for 250 days. Following eight days of habituation, the test trial procedure was started. Seven experimental and five controls were run during a three week period. All <u>S</u>s were initially habituated to the apparatus following six hours of water deprivation. Each <u>S</u> was placed in the start box and allowed to move about freely. Artificially sweetened water was available in each of the three end boxes. This procedure was repeated daily until <u>S</u>s were drinking within three minutes after being placed in the start box. The <u>S</u>s then had 12 trials each day and all combinations (WBW, WBB, WWB, BWB, BWW and BBW) were randomly presented twice. The water reinforcement was available in the odd box only. On the first four days, there were six randomly determined forced choices where only the odd-colored box was open. Over the four days there was an equal number of black and white forced correct choices. If the <u>S</u> entered an incorrect box during any of the test trials, the doors of the two remaining boxes were lowered to prevent a double error in any one trial. The <u>S</u> was allowed 30 seconds to leave the incorrect chamber, afterwhich he was picked up at that point and that trial was ended. If the <u>S</u> left the incorrect chamber, that door was lowered and the <u>S</u> picked up after 20 seconds. When the <u>S</u> entered the correct box, that door was lowered and drinking was allowed for five seconds. A variable intertrial interval (ITI) was used (30 seconds to one minute). The acquisition criterion was

10 correct responses in 12 trials. The rate of habituation and the number of correct choices were recorded.

The Ss were kept on the pair-feeding condition for three weeks following Test 5. They were then weighed and sacrificed. Their approximate age at that time was 500 days, and they had thus been under the experimental diet condition from midlife to end of life expectancy. A bone density analysis was performed on these and all Ss that had died and been preserved during the completion of the study.

RESULTS

The physiological measures, including plasma protein levels and weights for intact pairs, were statistically analyzed using the Student's t test for matched pairs. The remaining test data were analyzed using the t test or analysis of variance for independent groups. Although the physiological data were analyzed using the matched pairs t test, the matching was disregarded in the analysis of the behavioral and learning data, since Ss from both treatments were lost during this part of the study. This permitted data from the remaining pair-member to be used, thus keeping as many degrees of freedom for the analyses as possible. The reason for using both methods of data analysis was that it was unlikely that these psychological tests would necessarily show differences between pair-members to a greater degree than among members in each treatment. Weight was the only criterion for matching. The subjects were placed in pairs so that the appropriate pair-feeding procedure could be carried out to test for physiological differences between pair-members. A minimum level of significance of ($p \leq .05$) was set prior to the study, and non-directional tabled values were used.

Physiological Test Results

The analysis of both plasma protein levels and weights was performed on data collected after the study has been in progress for 10 weeks and prior to the loss of any Ss. The plasma protein levels were significantly different ($t = 2.85$, df 9, $p \leq .05$), for matched pairs and as expected the experimental, low-protein Ss had lower levels. The weights between pair-members were significantly different ($t = 2.78$, df 9, $p \leq .05$), and as predicted, the experimental pair-member weighed less than his control.

An analysis of the data from the activity wheel test, used for general activity, showed no significant differences; however the adequate protein group did have a higher group mean than the protein restricted Ss.

A 2 X 3 (diet by speed) analysis of variance was used to analyze the rotarod, motor-coordination data (Figure 4). The results showed poorer performance for the experimental group by diet ($F = 28.37$, df 1, 13, $p < .001$) and speed ($F = 44.24$, df 2, 26, $p < .001$) with a significant interaction between diet and speed ($F = 3.79$, df 2, 26, $p \leq .05$).

Fifty percent of the protein restricted rats became emaciated in outward appearance during this investigation. It was noted at the time of sacrifice that seven experimental and three control Ss survived. A Chi-Square test showed that the differences between survival rates approached significance ($X^2 = 3.2$, df 1, $p \leq .10$).

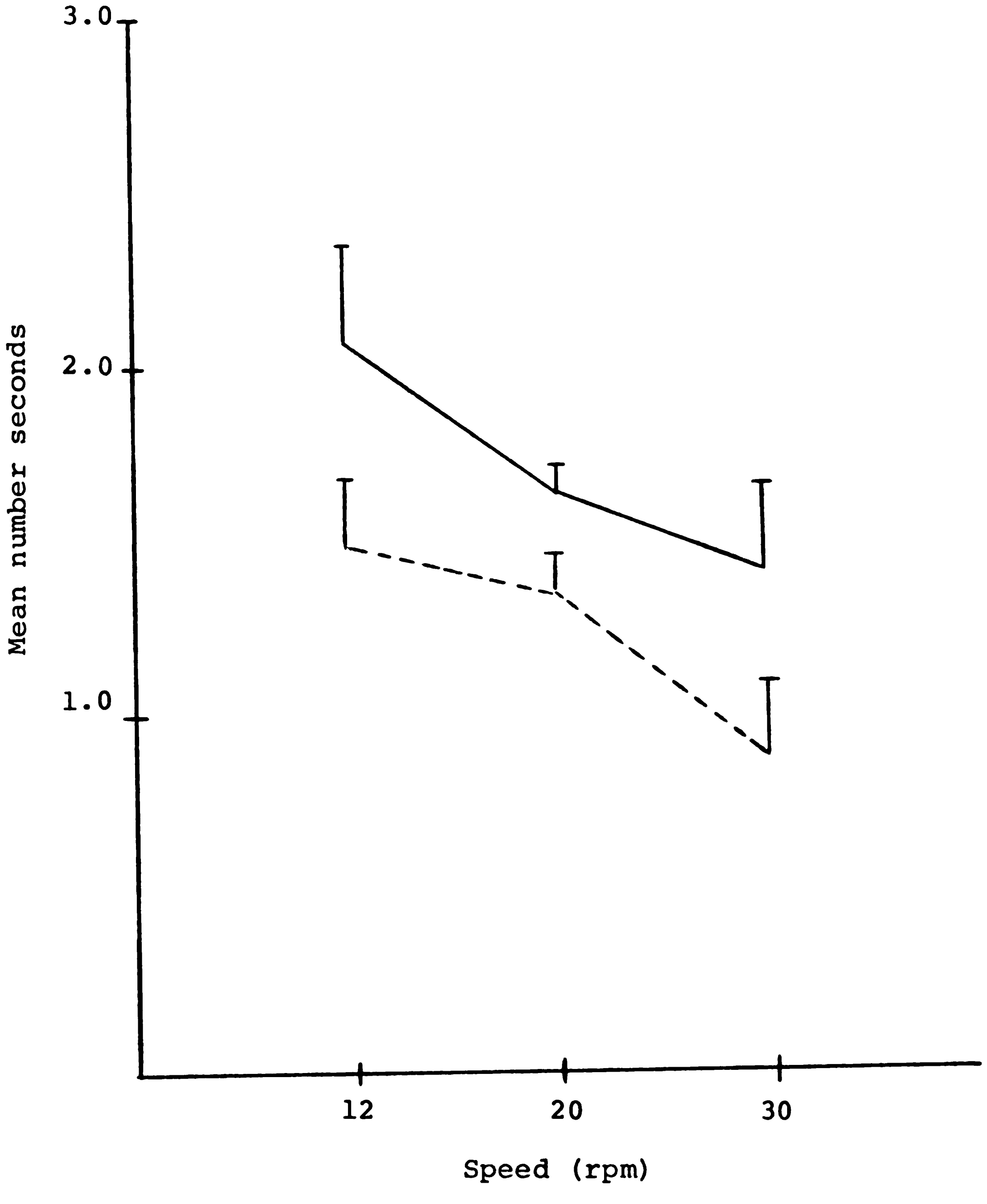

Figure 4. Mean number of seconds by speed for the experimental and control Ss on the rotarod, motor-coordination apparatus.

Behavioral Test Results

A one way analysis of variance was performed on the open-field test data (Table 2). Exploratory behavior was found to be significantly different between the experimental and control groups ($F = 5.01$, df 1, 14, $p \leq .05$), with the adequate-protein control Ss entering a greater number of squares than the low-protein, experimental Ss.

The food-preference data were analyzed for each group using Student t to test for a significant preference for the adequate-protein diet in both groups (Figure 5). This was found to be significant for the experimental group ($t = 7.102$, df 7, $p \leq .001$) and the controls ($t = 2.77$, df 6, $p \leq .05$). Both groups, when given a choice between diets preferred the 24 percent, adequate-protein mixture.

Learning Task Results

Two Ss from the experimental group would not run and therefore were dropped for purposes of data anlysis. It was noted that on day three of habituation the six controls were drinking within eight minutes after being placed in the apparatus (An eight minute time limit was preset as the maximum habituation time per day), while only three of the seven experimental Ss were drinking within that time. The differences in rate of habituation were not significant however, in general there was less exploratory behavior in the apparatus for the experimental group as compared to the controls. Since exploratory-approach behavior in a novel

Table 2. Performance of Male Holtzman Rats in an Open-field[a]

Diet	N	Numbers of Squares Crossed
Control	7	88.86 ± 9.00[b]
Protein-Restricted	9	65.88 ± 5.78[c]

[a]Animals were 320 days of age at testing and had been on respective diets for 120 days.

[b]Mean $\pm$ standard error.

[c]Significantly less than controls ($p < .05$).

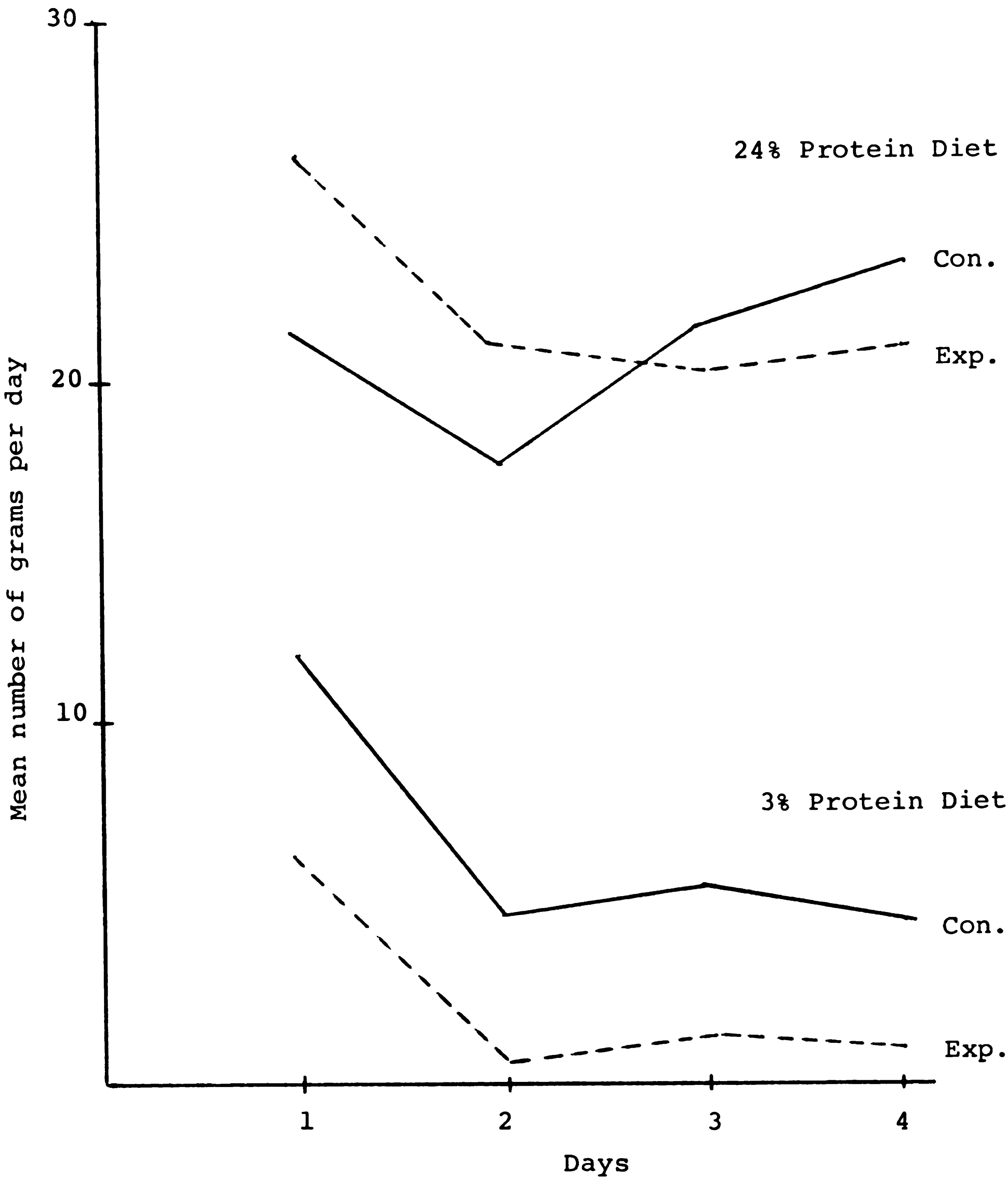

Figure 5. Mean number of grams per day for the experimental and control rats when offered both the 3 percent and 24 percent diets.

situation, e.g., the oddity-discrimination apparatus, is theorized to be part of an organism's innate behavior and a necessary initial component to learning a task, a lack of or diminished amount of such behavior is an important consideration (Denny and Adelman, 1955).

The data recorded during testing for the five of the seven experimental subjects that would run and the five controls were analyzed using the Student's t test. No significant differences were found between the groups for the number of correct choices made, however, the controls made fewer errors.

A strong preference for black end-box choice was noted to be present in both groups during habituation. A second t test was therefore performed to determine if this initial preference persisted throughout the study. It was found that the experimental group maintained a significant black preference ($t = 4.07$, df 8, $p \leq .01$) throughout testing, compared to the controls. This is further evidence that the experimental group had a tendency to avoid novel situations.

DISCUSSION

The general hypothesis of this study, stating that late-onset dietary manipulation can result in behavioral and physiological changes, was supported by the findings. Significant effects were found on the following measures: 1) plasma protein levels, 2) weights, 3) performance on the rotarod, 4) exploratory behavior, 5) food preference and 6) the number of white end boxes entered during the oddity discrimination learning task. Differences on the activity level measure, and number of errors on the oddity learning task were in the predicted direction but were not significant.

Since this was a preliminary study in the area of the relationship between nutrition and behavior in older organisms when the dietary insult occurs in later life, and is perhaps the first such study using an infra-human model, these results cannot be compared with many previous findings. A major concern in studies where behavioral problems and poor nourishment have been found to occur together, has been whether nutritional deficiencies cause the problems or result from them (Vinther-Paulsen, 1952; Loeb and Howell, 1970b, Schlenker et al., 1973; Whanger and Busse, 1975). The results of the present study suggest, that under certain circumstances, behavioral changes can result from dietary distress.

It was of great concern in this investigation to establish a methodologically sound research design in this area since poor methodology has been a major criticism of many of the perinatal nutrition-behavioral studies in the past (Greenfield and Briggs, 1971). Specifically, diet composition was well controlled, and the diets were made isocaloric. The pair-feeding procedure was used so that protein was the primary independent variable and was not confounded with caloric intake. All necessary nutrients were included in both diets, and drug therapy was not used for actue illnesses, since drugs have been found to interfere with metabolism of nutrient intake and behavior.

The methodological oversights in setting up this study included the failure to get baseline data for plasma protein levels and the failure to assess levels at the conclusion of the study. These data would have allowed a longitudinal comparison of the plasma levels of the experimental group, as well as the controls. Had levels become lower in the experimental group over time, this would have been good evidence that protein was the primary variable resulting in behavioral differences between the low-protein and adequate-protein groups.

If further research is to be continued in this area, it is pertinent to review some of the practical aspects of this investigation. The study demonstrated that older male Holtzman rats can survive extended feeding of meal diets and therefore can be kept on well controlled nutrient concentrations. The sensory capacity for taste and/or smell of these animals at approximately two thirds of their life expectancy, was sufficient to distinguish between the 3 percent low-protein diet and the 24 percent adequate-protein diet. This information indicates that food preference studies for older rats can be done. Confounding of age related variables with test results, such as occur with the increased probability of disease in older organisms, must be considered when determining the number of subjects to be used. It is necessary to establish the criteria for eliminating subjects that become ill during the study, prior to its start, e.g., those apparently unrelated to the experimental conditions.

Tests that have been used previously to assess behavior on various parameters have been used primarily with younger laboratory animals than those in this study. It is important, when testing naive older animals, that age-appropriateness of the tests be considered. If for instance, a fear response was elicited on the rotarod from these rats, this technique was not measuring motor coordination as was intended. When this can be determined or it is strongly suspected, an alternate test, such as the observation of locomotor performance on a narrow board, might be a more appropriate test for measuring motor coordination. Methods for determining age-appropriateness of tests are essential if specific behaviors are to be evaluated and should be determined for all tests used.

Further studies in this area, based on these findings, can be done using various nutrition deprivation models, e.g., protein calorie malnutrition model (PCM). The consideration of time needed to run an appropriate battery of tests may make it necessary to use a strain of rat with a longer life span than the Holtzman. This is especially true for learning tasks that may take hundreds of trials before differences can be detected. It can be predicted from previous nutritional research (Mills et al., 1969), that many nutritional models, e.g., PCM, trace element depletion, and various vitamin deficiencies can cause faster physiological effects than the protein-restricted model used here. If it can be demonstrated that behavior is also affected, using one of these faster models, it would be possible to design a study to include nutritive rehabilitation and subsequent repeated tests to see if reversal of effects would occur. It would, as evidenced by findings from various studies such as Barnes and co-workers (1966 and 1968), be advisable to do this type of study using both male and female rats to determine the presence or absence of sex differences.

APPENDICES

APPENDIX A

TEST DATA

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TEST DATA

Plasma Protein Levels at 10 Weeks

Blood samples were collected, plasma was separated from cells and analyzed. Eight of these analyses were repeated.

Subject Number	Grams/100 ml.
1	10.70
2	13.90
3	7.11[r]
4	7.04[4]
5	9.45
6	12.29
7	8.90
8	10.24
9	7.39[r]
10	7.60[r]
11	8.95
12	9.86
13	6.52[r]
14	6.79[r]
15	7.62
16	12.05
17	7.05
18	7.62
19	8.97[r]
20	9.16[r]

r indicates repeated analysis.

NOTE: Throughout appendices all odd numbered Ss refer to those under the experimental condition, and all even numbered subjects were controls.

Weights for all Ss at 10 weeks from beginning of the experiment and prior to the start of testing. These weights have been corrected for initial differences between pair-members.

Subject Number	Weight in Grams
1	405
2	410
3	462
4	436
5	501
6	506
7	501
8	502
9	471
10	514
11	487
12	547
13	344
14	416
15	528
16	552
17	490
18	496
19	452
20	528

Motor coordination

Experimental Ss by speed (N=8)

R_1	R_2	R_3
1.4	1.2	0.8
0.9	1.2	0.6
1.4	1.4	0.9
1.3	1.4	1.0
1.5	1.2	0.7
1.7	1.4	1.3
1.7	1.5	1.0
1.6	1.4	0.6

Control Ss by speed (N=7)

R_1	R_2	R_3
2.1	1.6	1.0
1.8	1.5	1.1
2.1	1.5	1.4
2.6	1.7	1.5
2.0	1.7	1.9
1.7	1.6	1.4
2.2	1.7	1.5

Open-Field

Experimental _S_s (N=9)

Number of Squares Entered
60
46
76
66
63
65
72
102
43

Control _S_s (N=7)

Number of Squares Entered
63
117
96
86
118
57
85

Food Preference

Experimental mean number of grams intake for each of four days when offered both diets (N=8)

Day	3 Percent	24 Percent
1	6.250	26.625
2	0.375	21.250
3	1.000	20.375
4	0.875	21.000

Control mean number of grams intake for each of four days when offered both diets (N=7)

Day	3 Percent	24 Percent
1	12.429	21.571
2	4.857	17.857
3	5.429	21.571
4	4.286	23.429

Oddity Discrimination

Number of correct responses for the experimental and control rats (120 trials N=5)

Experimental	Control
47	46
35	47
33	46
47	51
50	50

Number of white end boxes entered by experimental and control rats (120 trials N=5)

Experimental	Control
13	50
36	56
22	29
21	50
12	47

NOTE: For these analyses, two experimental animals were dropped since they would not habituate to the testing apparatus.

APPENDIX B

DESCRIPTION OF CONTENTS OF DIARY KEPT DURING STUDY

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A diary of notations was kept daily from the beginning of the experiments until the beginning of the last test (oddity discrimination) at which time notes were made, when necessary, on the raw data sheets. Recordings included _E_'s impression of each rat's general physical condition and behavior. Specifically anorexia, fluid intake, hair loss, appearance of stools, general activity, signs of chronic respiratory distress (CRD), acute and chronic illness and handling difficulties, were noted.

APPENDIX C

INFORMATION ON SPECIFIC SUBJECTS

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INFORMATION ON SPECIFIC SUBJECTS

Three controls within the first week of the experimental condition (pair-feeding) showed signs of acute-onset illness including anorexia and loose stools. A notation was made at that time that adjustment to the 24 percent diet needed to be considered as the possible cause. Two of these animals recovered, however number 8, a control rat, remained intermittently ill throughout the investigation until his death at 353 days of age. It was for this reason that this subject was omitted from all data analyses.

The procedure used for animals that became ill during the study was to isolate immediately for overt CRD symptoms while keeping all other variables approximately the same as under non-isolated conditions.

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